Rapid Method for Total Carotenoid Detection in Canary Yellow-Fleshed Watermelon

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ABSTRACT: Yellow-fleshed watermelons (*Citrullus lanatus* [Thunb.] Matsum. and Nakai) contain many different carotenoids, all in low to trace amounts. Since there is not 1 predominant carotenoid in yellow-fleshed watermelon, testing the total carotenoid content among watermelon lines is important in determining the antioxidant potential and thus potential health benefits of different varieties. Unfortunately, current methods to assay total carotenoid content are time consuming and require organic solvents. This report describes a rapid and reliable light absorption method to assay total carotenoid content for yellow-fleshed watermelon that does not require organic solvents. Light absorption of 78 watermelon flesh purees was measured with a diode array xenon flash spectrophotometer that can measure actual light absorption from opaque samples; results were compared with a hexane extraction method. The puree absorbance method gave a linear relationship ($R^2 = 0.88$) to total carotenoid content and was independent of watermelon variety within the total carotenoid concentration range measured (0 to 7 μg/g fresh weight).

Keywords: carotenoid, *Citrullus lanatus*, quantification, rapid assay, watermelon

Introduction

Carotenoids have been linked to lower risk of myocardial infarction (Kohlmeier and others 1997), may possess anticancer properties (Gerster 1997), and promote healthy eye function (Stahl and Sies 1996; Simon 1997; Giovannucci 1999; Rodriguez-Carmona and others 2006). These attributes may be, in part, responsible for increased purchase of products containing these compounds (McBride 1999; Natl. Watermelon Promotion Board 1999). Red-fleshed watermelons contain high quantities of lycopene, a red pigmented carotenoid with powerful antioxidant properties (Tomes and others 1963; Di Mascio and others 1989). Orange-fleshed watermelons typically contain high amounts of prolycopene, similar to the "tangerine" tomato mutation (Isaacson and others 2002), and in some varieties β-carotene and ζ-carotene are present (Tomes and Johnson 1965; Tadmor and others 2004, 2005; Perkins-Veazie and others, 2006). Yellow-fleshed watermelons are divided into 2 major types: canary yellow and salmon yellow (Henderson and others 1998). The salmon yellow is a "tangerine type" watermelon that contains small amounts of prolycopene whereas the canary yellow contains multiple carotenoids, all in low or trace amounts (Tadmor and others 2004, 2005). Tadmor and others (2004) reported that lutein and β-carotene were detectable in canary yellow-fleshed fruit.

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Carotenoid content varies greatly among watermelon cultivars and production environments (Perkins-Veazie and others 2001, 2006). Total carotenoid content was tested for Early Moonbeam, a canary yellow-fleshed watermelon (Henderson and others 1998) and only trace amounts of carotenoids were detected (Tadmor and others 2004, 2005). Tadmor and others suggest that this may be similar to tomatoes where a nonfunctioning phytoene synthase gene results in the accumulation of very low levels of carotenoids in canary yellow-fleshed fruits (Carmara 1993). However, since visual correlation of total carotenoid content is not accurate (Perkins-Veazie and others 2001), canary yellow-fleshed watermelon germplasm containing high concentrations of carotenoids may have been overlooked. Because carotenoids are valued as phytonutrients, a quick reliable screening method for carotenoid content is needed for germplasm evaluations. Conventional spectrophotometric or HPLC assays to quantify total carotenoids utilize organic solvents to extract and solubilize these compounds from tissue (Beerh and Siddappa 1959; Adsule and Dan 1979; Sadler and others 1990). These methods are time consuming, require the use and disposal of hazardous organic solvents, and do not work well for the more polar carotenoids. A simple, inexpensive, and reliable method to determine lycopene content in fruit was reported by Davis and others (2003a, 2003b); this method utilizes a diode array xenon flash spectrophotometer to measure absorbed visible color and correlates these values to lycopene content. In this report, we demonstrate that watermelon puree evaluated with a diode array xenon flash spectrophotometer is a novel and fast method for accurately quantifying total carotenoid content in canary yellow-fleshed watermelon. This method potentially overcomes detection problems with low carotenoid levels and the missing of more polar carotenoids.

Materials and Methods

Watermelon fruit

Ripe watermelons ranging from yellow to off-white were grown at Lane, Okla., U.S.A., in 2005. Ripeness was estimated by flesh texture and by soluble solids content measured from heart tissue using an

Atago PR100 digital refractometer (Bellevue, Wash., U.S.A.). Four open-pollinated varieties (Early Moonbeam, Yellow Baby, Yellow Doll, and Cream of Saskatchewan), 1 hybrid (Sunshine), 1 triploid (Tri-X Chiffon), and 5 Plant Introduction (PI) lines (271773, 271769, 299378, 314655, and 494531) were evaluated. Watermelons were stored uncut at room temperature from 0 to 7 d before heart tissue was removed for sample preparation.

Sample preparation

All steps from the time watermelons were cut lengthwise were performed in subdued lighting at room temperature. The heart tissue was collected and cut into approximately 3-cm^3 chunks or smaller. Immediately after collecting heart tissue, the samples were frozen and stored at −20 °C from 2 to 30 d, except for samples shown in Figure 4 where fresh samples were run and then rerun after freezing and thawing. Tissue (approximately 30 g) was homogenized using a Brinkmann Polytron Homogenizer (Brinkmann Instruments Inc., Westbury, N.Y., U.S.A.) with a 20-mm O.D. blade to produce a uniform slurry with particles smaller than 3 mm^3. This required continuous homogenizing for approximately 30 s. The samples were not allowed to heat or froth.

Low-volume hexane extraction method

The low-volume hexane extraction method, performed as described by Fish and others (2002), was used to measure total carotenoid content of watermelon purees. Approximately 0.6-g samples were weighed from each puree into two 40-mL amber screw-top vials containing 5 mL of 0.05% (w/v) BHT in acetone, 5 mL of 95% ethanol, and 10 mL of hexane. Purees were stirred on a magnetic stirring plate during sampling. The samples were placed on ice on an orbital shaker at 180 rpm for 15 min. After shaking, 3 mL of deionized water were added and the samples were shaken for an additional 5 min on ice. Vials were left at room temperature for 5 min to allow for phase separation. The absorbance of the upper, hexane layer was measured at 450 nm in a 1-cm path length quartz cuvette blanked with hexane. Total carotenoid content of watermelon was calculated based on sample weight using the absorbance at 450 nm (Wrolstad and others 2004). A comparison was made between the hexane extraction, methanol extractions, and ethanol extractions for each variety and PI, to ensure that the hexane method was adequately extracting all carotenoids (Wrolstad and others 2004). Additionally, the aqueous phase of the hexane extraction was scanned to ensure that all carotenoids were extracted into the hexane layer. This step was deemed necessary since the carotenoid profile of all yellow-fleshed watermelons is not known, and it was necessary to determine that more polar carotenoids were not underrepresented.

HPLC analysis

A subset of the samples tested above (16 samples of 5 varieties) and extracted as above with the low-volume hexane extraction methods were analyzed by HPLC. The samples were filtered using 0.45-μm PTFE syringe filters (Daigger, Vernon Hills, Ill., U.S.A.) into 2-mL amber crimp-top vials (Daigger, Vernon Hills, Ill., U.S.A.), then loaded onto a high-performance liquid chromatograph (HPLC) equipped with autosampler, photodiode array detector, and integration software (Hewlett Packard 1100,Wilmington, Del., U.S.A.). A C30 YMC carotenoid column (4.6 × 250 mm) and a YMC carotenoid guard column S-3 (4.0 × 20 mm) (Waters, Milford, Mass., U.S.A.) were used. A gradient method with 3 solvent mixtures was used to separate deionized carotenoids. Solvent mixtures of (A) 90% methanol, 10% deionized water containing 0.5% triethylamine and 150-mM ammonium acetate, (B) 99.5% 2-propanol, 0.5% triethylamine, and (C) 99.95% tetrahydrofuran, 0.05% triethylamine were applied as follows: initial conditions 90% solvent A plus 10% solvent B; 24-min gradient switched to 54% solvent A, 35% solvent B and 11% solvent C; final gradient conditions were 11-min gradient of 30% solvent A, 35% solvent B, 35% solvent C, held for 8 min. The mobile phases were returned to initial conditions for 15 min. Injection volumes of 100 μL were used for samples and standards. Standards ß-carotene, lycopene, phytoene, lutein, and phytofluene obtained from Sigma (St. Louis, Mo., U.S.A.) and violaxanthin, ζ-carotene, γ-carotene, and neoxanthin from Carotenature (Geneva, Switzerland) were used to attempt peak verification and ß-carotene and the ß-carotene extinction coefficient were used to calculate concentrations following the method of Craft in Wrolstad and others (2004).

Instrumentation

The UltraScan XE (Hunter Associates Laboratory Inc., Reston, Va., U.S.A.), a diode array xenon flash colorimeter/spectrophotometer, has a wavelength range from 360 to 750 nm that reads reflectance and transmittance. All tristimulus integrations are based on a triangular bandpass of 10 nm and a wavelength interval of 10 nm. The instrument sensor uses a specially coated plastic integrating sphere (6-inch diameter) that diffuses light from the xenon light source. The light source illuminates and is transmitted through the sample. A lens is located at an angle of 8° perpendicular to the sample surface that collects the transmitted light and directs it to a diffraction grating that separates the light into its component wavelengths. The intensities of these component wavelengths of transmitted light are then measured by 2 polychromators each with 40-element diode array detectors.

Puree absorbance method

The instrument was standardized as per company specifications and blanked on a cuvette filled with deionized water. Watermelon puree was mixed well to keep separation to a minimum; about 20 mL of the sample was immediately poured into a 1 cm, 20-mL SR101A cuvette (Spectrocell, Oreland, Pa., U.S.A.). The sample was scanned in the transmittance (TTRAN) mode under the following settings: the large reflectance port (1.00 inch), Illuminant at D65, MI Illuminant Fcw, and observer 10°. Absorbance at 670 nm was subtracted from absorbance at the maximum absorbance (430 nm) to adjust for light scatter. The low-volume hexane analysis and the puree absorbance readings were performed on the same day to avoid possible storage effects on the samples.

To achieve the desired level of reliability in the puree absorbance procedure, one must (1) maintain subdued light since light degrades carotenoids, (2) thoroughly homogenize the tissue, and (3) mix the puree before reading the samples.

Statistical analysis

Linear least square regression analyses were performed using the statistical component of Microsoft® Excel 2002 SP-2 software (Redmond, Wash., U.S.A.).

Results and Discussion

Absorbance spectral measurements of watermelon tissue puree

Previous attempts at reliably correlating reflectance parameters to carotenoid content of watermelon with handheld colorimeters were unsuccessful (Perkins-Veazie and others 2001; unpublished results). Use of tissue puree can overcome this limitation as well as provide access to light absorption to provide a reliable quantification method. The Hunter UltraScan XE subjects samples to light

intensities that are orders of magnitude greater than those of analytical spectrophotometers with quartz halogen lamps. The UltraScan XE also has sphere collectors that collect scattered light as well as nonscattered light. This potentially allows reliable spectral measurements on translucent samples that scatter light. The spectra for yellow-fleshed watermelons exhibit apparent absorption maxima of 410, 430, 450, and 480 nm (Figure 1). These maxima are decidedly different from the maxima around 381, 401, 426, and 448 that are observed for canary yellow-fleshed watermelon samples in organic solvents such as hexane. These absorption peaks are missing from or hidden in the spectrum of purees from red- and orange-fleshed watermelon, which contain predominately lycopene and prolycopene, respectively.

Absorbance behavior of watermelon puree as related to total carotenoid content

Based on the spectral results obtained from the UltraScan XE, we investigated the possibility of employing absorbance measurements of watermelon puree at 430 nm as a means to estimate total carotenoid content of watermelon tissue. Since many purified carotenoids are not soluble in an aqueous phase, and because the carotenoid profile of yellow-fleshed watermelon is not fully known, a standard curve with purified carotenoids could not be performed. Therefore, visible absorbance of watermelon puree using the UltraScan XE was correlated with total carotenoid content determined by absorption measurement of organic solvent extraction. Because the carotenoid profile of yellow-fleshed watermelon is not well characterized, we tested 2 fruit from each variety using the methanol and ethanol extraction methods (Wrolstad and others 2004) and compared them to the hexane method described above. Of the 3 methods, the hexane method gave the most reproducible and the highest estimate of total carotenoids in all samples (data not shown). This suggested that the carotenoids present were highly soluble in hexane and thus this solvent was used for this study.

Flesh tissue from 132 yellow- to off-white-fleshed watermelons from 6 varieties and 4 PIs were used to develop an equation to predict total carotenoid content. The absorbance at 430 nm measured for each puree (adjusted for light scatter by subtracting the absorbance at 670 nm) was plotted against its total carotenoid content as measured by hexane extraction (Figure 2). The scatter-adjusted absorbance at 430 nm appears to obey Beer's law with respect to total carotenoid content. The absorbance reading is linearly correlated with total carotenoid content, and the linear least squares fit to the data yields the equation $y = 0.2806x + 0.28$ with an R^2 value of 0.88. To predict total carotenoid content, one must solve for x yielding the following equation: $x = (y - 0.28)/0.2806$.

The precision of the UltraScan XE assay procedure appears to be comparable to that of the conventional assay. For 78 randomly chosen watermelon samples assayed by the puree absorbance method, the average standard deviation per duplicate readings (430 to 670 nm) was ± 0.01 absorbance or ± 0.01% average standard

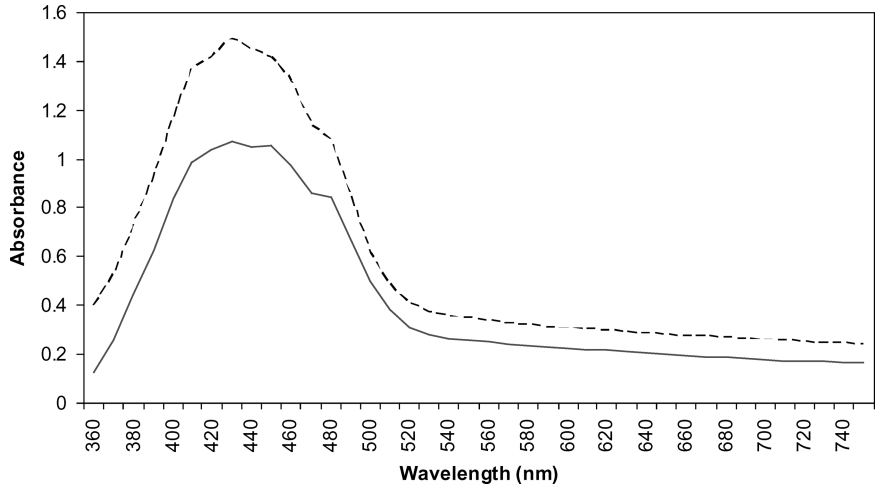

Figure 1—Visible absorption spectra from a Hunter Lab UltraScan XE of 2 yellow-fleshed watermelon purees of differing total carotenoid content. Both samples were from "Sunshine" watermelons; 1 contained 3 μg/g total carotenoids (dashed line) and the other contained 2 μg/g total carotenoids (solid line). Total carotenoids were determined as described under Materials and Methods.

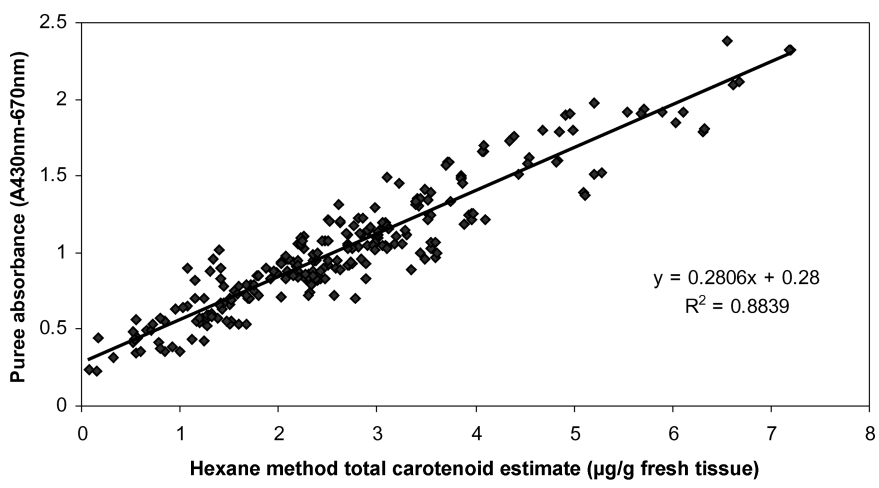

Figure 2—Absorbance of 132 yellow-fleshed watermelon heart tissue purees compared with total carotenoid content. Absorbance of duplicate samples analyzed with the UltraScan XE, is plotted compared with total carotenoid content as determined by duplicate hexane extractions for a total of 264 points on the figure. The absorbance at 430 nm is adjusted for scatter by subtracting the absorbance at 670 nm. The R^2 value and the linear least squares fit equation are given in the figure.

error. The average standard deviation and standard error per duplicate sample for all 78 purees with the low-volume hexane method was ± 0.001 and ± 0.02%, respectively. The method reported here is as precise as the currently accepted hexane extraction/absorbance methods (Beerh and Siddappa 1959; Adsule and Dan 1979; Sadler and others 1990).

Use of watermelon puree absorbance to quantify total carotenoid content

To validate the equation obtained in Figure 2 as a predictive equation for total carotenoid content in watermelon tissue, the scatter-adjusted absorbance (430 to 670 nm) of 45 additional watermelon purees (3 varieties and 3 PI lines) was measured. Then, each value was inserted into the linear equation generated by Figure 2 to estimate the total carotenoid content of the tissue. Estimated total carotenoid contents from the puree absorbance method were plotted against the total carotenoid values estimated by hexane extraction (Figure 3). A linear relationship was obtained between the estimates by the 2 methods with an R^2 of 0.91. The equation for the linear least squares fit to the data, $y = 0.9229x + 0.2802$, differs only slightly from that expected for an ideal fit, $y = x$.

Yellow watermelon samples demonstrated from 3 to 14 different carotenoid peaks per variety when analyzed by HPLC. Attempts to identify these by comparing the available standards and know published carotenoid peaks proved difficult. Most of the carotenoid peaks appeared to be esters, because on saponification almost all the HPLC peaks in the nonpolar part of the chromatogram disappeared and were replaced by new peaks with shorter retention times. Saponification of 1 yellow watermelon sample with 10% KOH and an antioxidant (BHT) in methanol for 3 h demonstrated 9 carotenoid peaks. Four were tentatively identified as violaxanthin, luteoxanthin, auroxanthin isomer, and mutatoxanthin (Harold Furr, Craft Laboratories, personal communication). Unfortunately, saponification degrades many carotenoids (Kimura and others 1990; Larsen and Christennsen 2005). A total of 4 saponified samples demonstrated approximately 33% to 50% less carotenoid content than the samples that were not saponified. Therefore, we calculated carotenoid content using the values obtained from HPLC runs on the samples that were not saponified. These values were compared to the same samples run using the low-volume hexane method. Our results showed that the 2 methods are comparable (data not shown) with a standard deviation of ± 0.2 μg/g.

Absorbance behavior of fresh and freeze-thawed watermelon puree as related to total carotenoid content

Since Fish and Davis (2003) demonstrated that a freeze–thaw cycle caused a decrease in lycopene levels in pureed red watermelon samples, we wanted to determine if the same occurred to total carotenoid content in yellow-fleshed samples. Figure 4 is a

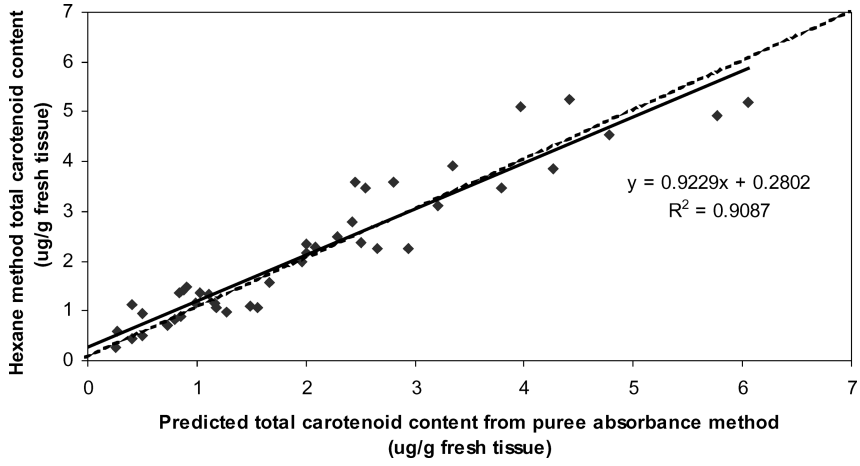

Figure 3—Total carotenoid content determined for 45 individual yellow-fleshed watermelons by the puree absorbance method as compared with the determinations by hexane extraction. The adjusted absorbance at 430 nm (that is, 430 minus 670 nm) for each watermelon puree was converted to a total carotenoid content with the use of the linear least squares equation generated in Figure 2. Total carotenoid estimates were obtained by solving for $x = (y - 0.28)/0.2806$. The R^2 value and the linear least squares fit equation are given in the figure. The hatched line indicates a perfect predictive model, that is, $y = x$.

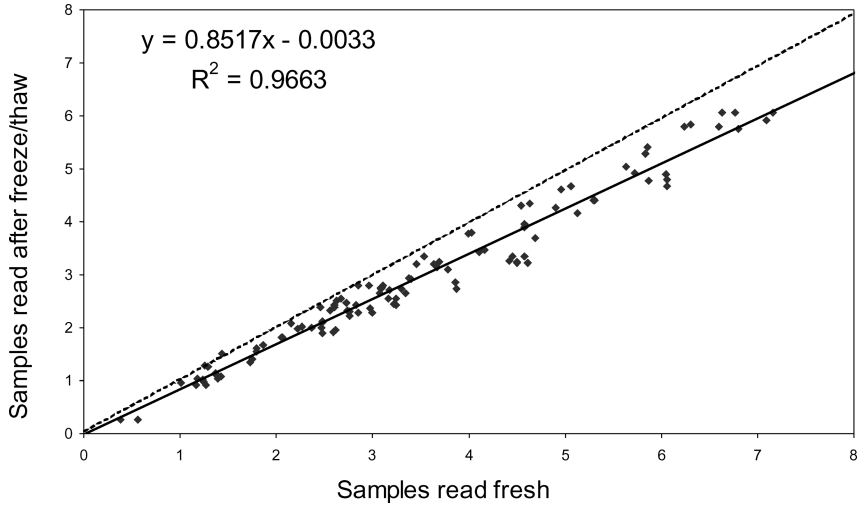

Figure 4—A comparison of estimated total carotenoids, expressed in μg/g fresh tissue, for 54 canary yellow-fleshed watermelon samples read fresh and after freeze thawing. Duplicate samples are shown for a total of 108 points on the figure. The hatched line indicates a perfect predictive model, that is, $y = x$.

comparison of 54 freshly pureed watermelon samples compared to the same samples put through a freeze–thaw cycle. The absorbance readings of the samples were less (an average decrease of 15%) after thawing than when the same samples were read fresh. The percent decrease in the absorbance after freeze–thaw varied around 15% throughout the range of total carotenoid content measured. We were unsure whether our decrease in absorbance after a freeze–thaw cycle was due to a change in the matrix of the sample causing a difference in absorbance, or if it was an actual decrease in the carotenoid content. To test this, we compared 5 pureed yellow-fleshed watermelon samples using the hexane method on both fresh tissue and after thawing (data not shown). These results showed no significant decrease in total carotenoid content in the thawed samples. This suggests that the decreased absorbance in the thawed puree samples is likely due to a change of puree matrix rather than a diminution in total carotenoids.

It is interesting to postulate on the difference between loss of lycopene in red-fleshed watermelon and the seeming stability of yellow-fleshed watermelon. It could simply be a sensitivity issue, since yellow-fleshed watermelons have an order of magnitude less total carotenoids than red-fleshed fruit. However, it may be that the configuration of the carotenoids in their chromoplast, or the carotenoids themselves, are more stable in yellow than in red watermelon.

To estimate total carotenoid content from fresh canary yellow-fleshed watermelon samples using the puree absorbance method, fresh tissue will need to be used to generate the standard curve. If estimating total carotenoid content on tissue that has been frozen and thawed using the puree absorbance method, the standard curve will need to be generated with tissue that has undergone a freeze–thaw cycle, as in this study. The resultant equations generated for the 2 forms of tissue will not be interchangeable.

Until it is determined that all xenon flash colorimeter/spectrophotometers give identical responses to given levels of total carotenoid contents, a response curve like that in Figure 2 will have to be generated for each instrument.

Conclusion

This article describes a simple, rapid, and cost-effective method to quantify total carotenoids in flesh of canary yellow-fleshed watermelons. We evaluated 177 watermelon samples (6 varieties and 5 PI lines) with total carotenoid contents ranging from 0 to 7 μg/g fresh weight. The puree absorbance method gave a linear relationship to carotenoid content and is independent of watermelon variety. This method offers an improvement over conventional methods by reducing sample processing time by at least half and requires no hazardous reagents. It is important to note that this equation may have to be altered for yellow-fleshed fruit that have a higher total carotenoid than tested here (>7 μg/g). Until this germplasm is found, if it indeed exists, this method is a good screening tool to search for canary yellow-fleshed watermelon germplasm with high carotenoid content.

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